INDUCTION OF APOPTOSIS BY EPIGENETIC MODULATORS IN HUMAN BREAST CANCER AND EXPRESSION PROFILE OF EZH2 AND RAS SIGNALLING COMPONENTS

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CERTIFICATE

This is to certify that the thesis entitled "**INDUCTION OF APOPTOSIS BY EPIGENETIC MODULATORS IN HUMAN BREAST CANCER AND EXPRESSION PROFILE OF EZH2 AND RAS SIGNALLING COMPONENTS**" which is being submitted by **Mr. Dibyojyoti Baruah**, Roll No. 411LS2042, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by him under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that this project report on, **"INDUCTION OF APOPTOSIS BY EPIGENETIC MODULATORS IN HUMAN BREAST CANCER AND EXPRESSION PROFILE OF EZH2 AND RAS SIGNALLING COMPONENTS"**, is the result of the work carried out by me .Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of ***Dr. Samir Kumar Patra, Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela.***

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ABSTRACT

Apoptosis is a process which self-destroys and removes unwanted cells in a body. It takes place to maintain a constant cell numbers and to balance the cell proliferation. Additionally apoptosis provide a defense mechanism by which damaged and potentially dangerous cells get eliminated from the body. Similarly it gets activated when cell has undergone DNA damage and as a result of which it induced apoptosis. Control of gene expression is exerted at a number of different levels, one of which is the accessibility of genes and their controlling elements to the transcriptional machinery. Accessibility is dictated broadly by the degree of chromatin compaction, which is influenced in part by polycomb group proteins. EZH2, together with SUZ12 and EED, forms the polycomb repressive complex 2 (PRC2), which catalyzes trimethylation of histone H3 at lysine 27 (H3K27me3).This study was aimed to defined the oncogenic role of EZH2 and Ras signaling components and it role in induction of apoptosis. The study was performed to know the role played by epigenetic modulators 5 Aza 2`deoxycytidine (AZA) and Trichostatin A (TSA) on MDA MB-231 cells and the changes takes place after adding these epigenetic modulators.

INTRODUCTION

In an adult human being all kind of cells contain the same genetic material. But astonishingly these cells perform different function in different parts of the body. This fate of the cells is decided by the initial expression of their gene and after the fate and position of the cells are decided, than their identity is preserved by keeping some genes on and others off**.** If the maintenance mechanism fails, the cells whole functions get change by losing their properties of proliferation, differentiation, adhesion or invasion etc. which cause a disease which we all known as by the name of cancer [1, 2].Cancer is a disease that cause from the defects in maintaining the original cellular memory, causing cells to loss its normal function and react inappropriately. The Polycomb Group (PcG) and Trithorax Group (TrxG) have been identified to keep the cellular memory and ensures a faithful transmission of information for which gene has to be active or repressed from mother to daughter cells [1, 3]. They execute the process through epigenetic modification like DNA methylation, histone modification and chromatin remodeling to complete their normal function [4, 5].

For maintaining and establishing the original cellular identity, many pathways are involved in repressing or activating particular sets of genes. The genes of Polycomb group and trithorax group are conserved from Drosophila to mammals and they maintain the same transcription memory patterns which are established during the early stages of embryonic life, and are continued from one cellular generation to the next. The TrxG and PcG proteins are two antagonistic groups which are required for the regulation of genes related to development and cell cycle through activation and repression respectively. As chromatin contains the imprints underlying the cellular memory and epigenetic inheritance, both multimeric complexes can act on their target genes by modulating chromatin structure. In Drosophila and mammals, both TrxG and PcG bind to a specialized DNA element, Polycomb/Trithorax Response Elements [PREs/TREs] to perform their epigenetic function [6].

By genetic screening, TrxG and PcG were initially identified in Drosophila to establish and maintain homeobox (Hox) gene expression patterns [7-9]. But the function of the TrxG and PcG

is not limited to regulate Hox gene expression [10]. Further studies have shown that these proteins bind to thousands of chromosomal site in addition to the Hox genes [8, 9].

The PcG proteins comprise of multiprotein complexes, which appear to perform their respective functions. Two distinct Polycomb complexes have been characterized by immune precipitation, yeast two hybrid and size-fractionation experiments in mammalian system. The Polycomb Repressive Complex 1 (PRC1) constitute BMI-1, RING1, HPH1/2/3, and HPC1/2/3 (Psc, dRING, ph and Pc in Drosophila) proteins [5, 11] and EZH2, EED, SUZ12, RbAp46/48 and AEBP2 (E(z), Esc, Su(z)12, and RbAp48 in Drosophila) [5, 9, 11] make the Polycomb Repressive Complex 2 (PRC2).Polycomb Repressive Complex 2 (PRC2) plays a vital role in the chromatin modification by di- and tri-methylation of Lys 27 of Histone H3 (H3K27me2/3) [5, 12-14]. The high level of H3K27me3 transforms the genes to a silenced state and transcriptional repression in higher eukaryotes.

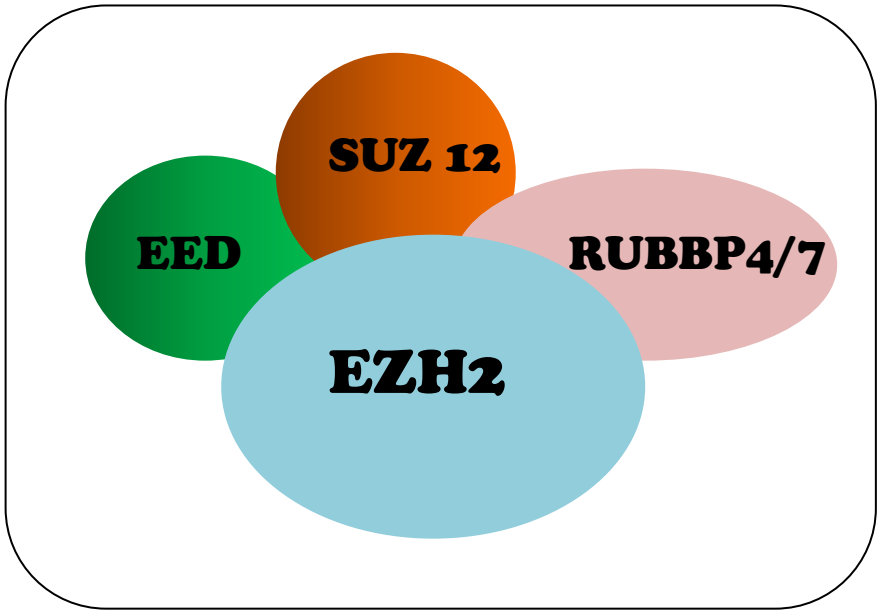


Fig 1: Components of PRC2

COMPONENTS OF PRC2

The four core proteins of PRC2 are conserved from *Drosophila* to humans and it's weigh about 230 KDa [15]. The EED protein which stands for embryonic ectoderm development is a human homolog for Esc protein of *Drosophila.* It is a short protein of 425 amino acids that

contain five WD40 repeats, shown to form a β propeller structure [16]. The Suppressor of zeste 12 (SUZ12) protein is 900 amino acid long and characterized by a C_2H_2- type zinc finger and a carboxy-terminal VEFS domain [17]. The catalytic subunit of PRC2 protein is EZH2 which encodes a 760- amino acid protein containing a conserved SET domain that confers histone lysine methyl-transferase (HKMT) activity. In addition to the essential role of EED and SUZ12 in PRC2, histone-binding protein RBBP4 (or RbAp48/46) and the zinc finger protein AEBP2 bind to PRC2 and optimize its enzymatic activity in vitro [11].

Ezh2 activity appears to depend on interaction with both Suz12 and the WD40 domain in EED [11, 18]. EED's WD40 beta propeller, in turn, interacts with H3K27me3 repressive marks. This interaction is proposed to promote the allosteric activation of PRC2 methyltransferase activity [18]. RbAp48 also contains a WD40 propeller required for interaction with both Suz12 and the first 10 residues of unmodified Histone H3 peptides. Biochemical studies have shown that PRC2 co-purifies with the protein AEBP2 and it has been proposed that this interaction aids in targeting of PRC2 to specific DNA sites and enhances its methyltransferase activity [19]. This important cofactor is an evolutionarily conserved protein present in two isoforms in humans, an adult-specific larger form (51 kDa) and an embryo-specific smaller form (32 kDa), both containing three Gli-Krüppel (Cys2-His2)-type zinc fingers [20].

EZH2 and DNA Methylation

The polycomb group protein EZH2 directly controls DNA methylation. The essential epigenetic systems involved in heritable repression of gene activity are the Polycomb group (PcG) proteins [21, 22] and the DNA methylation systems [23, 24]. These two processes are not independent but dependent on each other for the repression of gene expression. Through chromatin immunoprecipitation technique it was indicated that the binding of DNMTs to several EZH2-repressed genes depends on the presence of EZH2 [4]. Furthermore it was revealed through bisulphite genomic sequencing that EZH2 is required for DNA methylation of EZH2-target promoters [4, 25]. Thus EZH2 serves as a recruitment platform for DNA methyltransferase (Figure: 2).

The PcG proteins are responsible for de novo DNA methylation in cancers or cancer cell lines [26]. Through ChIP analyses, it is revealed that genes having DNA methylation in cancer are marked with Polycomb proteins. It's already known that, repression of many tumor suppressors genes are done by de novo DNA methylation, and they are already pre-marked with H3K27 tri-methylation, indicating the mechanism by which EZH2 and PRC2 promote the progression of cancer. In normal tissues, some tumor suppressors are marked with H3K27 tri-methylation, but de novo DNA methylation is not reported, therefore it signifies that EZH2 alone cannot causes DNA methylation, but several other components are required methylate DNA for gene repression.

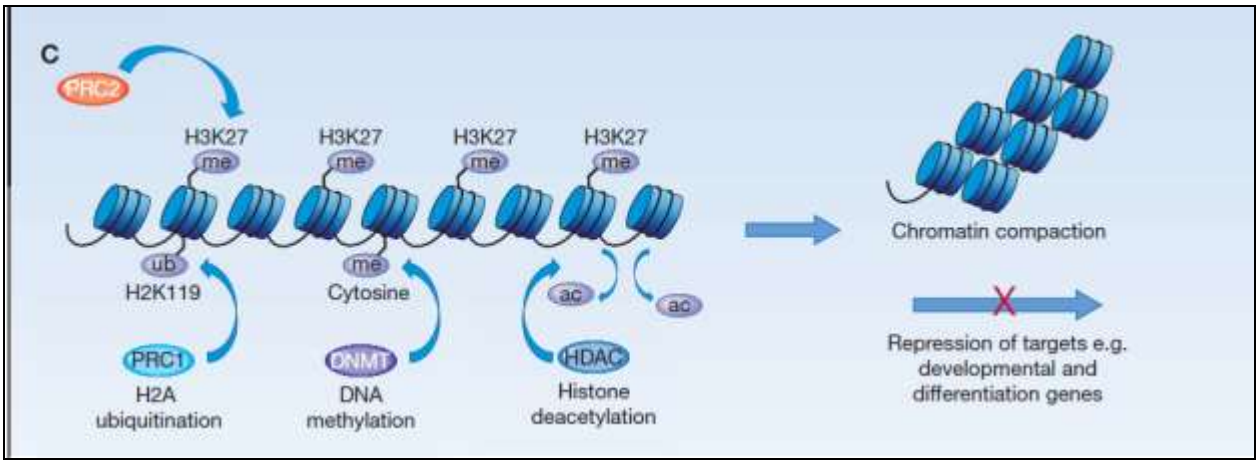


Fig 2: PRC2 is first recruited to DNA resulting in H3K27me3. This mark facilitates recruitment of PRC1, DNMT and HDAC. (Source: Aberration of EZH2 in cancer)[27]

EZH2 and Cancer

Through genome-wide analysis and various DNA methylation analyses, it has been reported that PRC2-mediated H3K27me3 as an epigenetic mark pathogenically which involved in cancer progression through silencing of tumor suppressor genes [28-31]. The expression and functions of EZH2 is altered in cancer cells and its overabundance is correlates with tumor aggressiveness and poor prognosis. EZH2 normally doesn't express in adult cells but it is overexpressed in a broad range of hematopoietic and solid human malignancies (Table 1), where its overabundance is often associated with poor prognosis. The overexpression of EZH2 was first reported in

aggressive and metastatic prostate cancer through gene expression profiling. In breast cancer, abnormally high levels of EZH2 are likewise associated with tumor aggressiveness, increased risk of metastasis and shorter patient survival. Increased cell proliferation is commonly associated with EZH2 overexpression in cancers. Conversely, loss of EZH2 inhibits growth of cancer cells.

TABLE 1:- Overexpression of EZH2 and its functions in human cancers.

TYPE OF CANCER	FUNCTION	REFERENCES
Prostate Cancer	Cellular Transformation Proliferation Invasion and Metastasis	[32, 33]
Breast Cancer	Cellular transformation Proliferation Invasion and metastasis	[34, 35]
Lymphomas	Proliferation	[36]
Bladder carcinoma	Cellular transformation Proliferation	[37]
Colon cancer	Proliferation	[38]
Hepatocellular carcinoma	Proliferation Invasion and metastasis	[39]
Pancreatic cancer	Proliferation Anti-apoptosis	[40]

Epigenetic Modulators

Human cancers result largely from the inappropriate silencing or activation of genes. It is well recognized that the expression of a gene can be controlled to some extent by modulating the access of the transcriptional machinery by targeting genes through chemical modifications of DNA sequences or histones. These modifications are done with the help of cellular enzymes, including DNA methyltransferases, histone acetyl transferase (HATs), histone deacetylases

(HDACs), histone methyltransferases (HMTs), histone demethylases, and histone kinases. Epigenetics enzymes plays an important role during the embryonic development by regulating differentiation pathways, and in adults life also they continue to play there role in specific tissues by maintaining epigenetic and transcriptional patterns and act as a co-regulator for transcription factor. During various cancers, these epigenetic enzymes loss their function and expressed abnormally as a result of which the transcriptional processes get altered which lead to the silencing of tumor suppressor genes or unchecked cellular growth [41].

For the last two decades, it has been tried to identify the drugs that can modulate the pathways mediated by epigenetic enzymes. However, till now only a few drugs have shown success in clinical trials, and others are toxic due to their unspecific effects on cellular function [42-44].To date, there are only a few epigenetic drugs which are approved by the FDA, including: 5-azacytidine which is a DNA methyltransferase inhibitor [45]. Another drug known as Trichostatin A (TSA), is an antifungal antibiotic having cytostatic and differentiating properties in mammalian cell culture, and also a potent and specific inhibitor of histone deacetylase (HDAC) activity [46, 47].

5-aza 2`-deoxycytidine (AZA)

5-aza 2`-deoxycytidine is an analog of cytosine residue which competes with cytosine for the active site of DNA methyltransferase (DNMT) (figure 3). When AZA is present inside a nucleus it inhibits DNA methylation which results in the loss of methylation level of specific genes and activation of that particular gene. Normally the catalytic mechanism of DNA methyltransferase start with a formation of a covalent bond between a cysteine (Cys) residue present in the active site of the enzyme and carbon 6 (C6) of cytosine (Cyt) in DNA. This increases the flow of electrons to carbon 5 (C5), with a subsequent attack on the methyl group of S-Adosyl Methionine(SAM).This led to the abstraction of a proton from C5 followed by β -elimination and reformation of the 5 = 6 double bond and release of the enzyme and DNA with a methylated Cytosine. Therefore AZA plays its inhibitory role by binding to the active site of DNMT which results in the decrease it activity. 5-Aza-2′-deoxycytidine, when get added into the genomic DNA in sites that are to be occupied by cytosine during replication, is best known as an inhibitor

of DNA methyltransferases (DNMTs—DNMT1, DNMT3A, andDNMT3B) through covalent adduct formation with the enzyme [48].

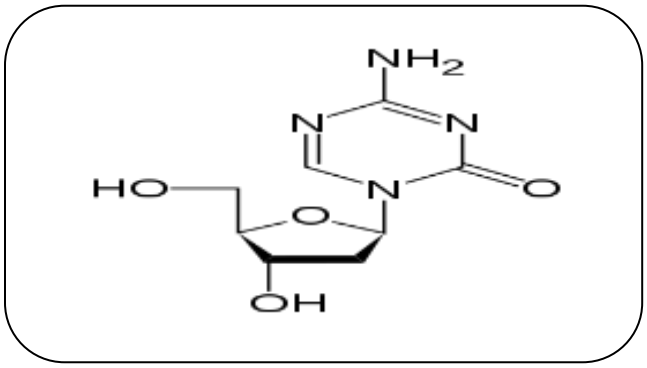


Fig 3: 5-Aza 2`-deoxycytidine

Trichostatin A

Trichostatin A (TSA) is an antifungal antibiotic with cytostatic and differentiating properties in mammalian cell culture and is a potent and specific inhibitor of histone deacetylase (HDAC) activity (Figure 4).

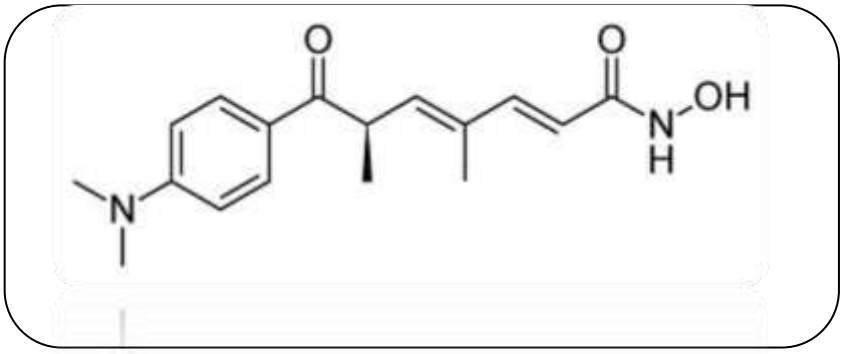


Fig 4: Trichostatin A

REVIEW OF LITERATURE

In mammals, two main Polycomb group complexes exist - Polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC1 compacts chromatin and catalyses the mono-ubiquitylation of histone H2A. PRC2 also contributes to chromatin compaction and catalyses the methylation of histone H3 at lysine 27. PRC2 is involved in various biological processes, including differentiation, maintaining cell identity and proliferation, and stem-cell plasticity [15].JNK-STAT3-Akt signaling axis to EZH2 phosphorylation to add novel pathway of carcinogenesis [49]. Simon and colleagues in the April 1, 2012, issue of Genes & Development, correlated disruption of Ezh2 in mice is sufficient to T-acute lymphoblastic leukemia (T-ALL) development and it was shown that a similar mechanism is involved in human T-ALL development [50]. EZH2 mediates transcriptional repression through the characteristic methyltransferase activity at the chromatin level has certain influence on lymphoma, and suggests a therapeutic window for the development of new agents and identification of EZH2based novel diagnostic markers [51].

Ezh2-KI mice develop myeloproliferative disorder, showing excessive myeloid expansion in bone marrow and spleen, leukocytosis and splenomegaly. Ezh2 plays critical role in in haematologic malignancies by regulating hematopoietic stem cell-specific genes such as Evi-1 and Ntrk3 that are often aberrantly expressed [52]. EZH2, a novel prognostic biomarker in NSCLC promotes progression and invasion of non- small cell lung cancer [53]. EZH2 implicates disease progression, at least partly through promoting cell growth, confers tumor aggressiveness. Thus it represents an independent adverse prognosticator in patients with NPC [54]. In triple-negative breast cancer, high enhancer of zeste homolog 2 expression is significantly associated with decreased survival [55].Increased expression of EZH2 does not necessarily correlate with increased abundance of H3K27me3 always to support the idea that EZH2 can have effects beyond epigenetic silencing of target genes in breast cancer [56]. Loss of heterozygosity affecting chromosome 7q is common in acute myeloid leukemia and myelodysplastic syndromes and this gives the importance of that region in diseased phenotypes and clonal evolution involving a homozygous EZH2 mutation [57]. PTEN and MYC exist in homeostatic balance to

control normal growth which is disrupted in cancer cells. MYC acts via the PTEN tumor suppressor and elicit auto-regulation with genome-wide gene repression by activation of the Ezh2 methyltransferase [58]. EZH2 has also role in nasopharyngeal carcinoma as it significantly enhanced the proliferation and invasion of nasopharyngeal carcinoma cells in vitro, which might be mediated by inducing EMT [59]. AXL receptor kinase expression is found in human gliomas with high EZH2 expression and drives glioma invasiveness via transcriptional control of AXL receptor kinase independent of histone or DNA methylation [60]. EZH2 is also shown to be associated with high grade and basal-like tumors [61]. EZH2 accelerates cancer cell invasion, in part, via RKIP(Raf-1 kinase inhibitor protein)inhibition and is involved in the regulation of RKIP transcription which suggests a potential mechanism by which EZH2 promotes tumor progression and metastasis [62].

Mutation, EZH2 and Cancer

Mutation of A677 in histone methyltransferase EZH2 in human B-cell lymphoma promotes hyper-trimethylation of histone H3 on lysine 27 (H3K27) [63]. EZH2 mutations appear to be critical in T-cell acute lymphoblastic leukemia development. In addition, the discovery of frequent mutations in , EZH2, CREBBP, EP300 and MLL2 in B-cell lymphomas suggests that epigenetic alterations play a critical role in lymphomagenesis [64]. Recently, four EZH2 single-nucleotide polymorphisms (SNPs) have been described in colorectal cancer (mCRC). One allelic variant (rs3757441 C/C versus C/T or T/T) was significantly associated with shorter progression-free survival (PFS) and overall survival (OS). At multivariant analysis, the same variant resulted an independent predictor of PFS and OS. The C/C variant was associated with significantly higher EZH2 expression. An EZH2 SNP may be useful to predict clinical outcome in mCRC patients [65]. In another study where eight matched tumor-constitutional DNA pairs from patients with sporadic parathyroid adenomas underwent whole-genome capture and high-throughput sequencing , revealed that one tumor harbored a Y641N mutation of the histone methyltransferase EZH2 gene, previously linked to myeloid and lymphoid malignancy formation. This implicates a previously unassociated methyltransferase gene EZH2, in endocrine tumorigenesis and therapy [66].A substantial proportion of patients with

myelodysplastic syndromes (MDSs) or chronic lymphocytic leukemia (CLL) harbor Spliceosomemutations , which are often missense in type. Such mutations are infrequent in other lymphomas, which instead display a separate group of novel mutations involving genes whose products are believed to affect histone acetylation and methylation and chromatin structure (for example, EZH2 and MLL2) [67].

EZH2 IN CANCER THERAPY

Overexpression of the Polycomb repressive complex2 (PRC2) subunit Enhancer of Zeste 2 (EZH2) occurs in several malignancies, including prostate cancer, breast cancer, medulloblastoma, and glioblastomamultiforme etc. EZH2 also enhances cancer-cell proliferation and regulates stem cell maintenance and differentiation. Henceforth, EZH2 targeting via various pharmacological interventions may proof beneficial in cancer therapy. Here we discuss some of the recent methods of targeting this epigenetic regulator in different cancers.

Current evidences suggest that EZH2 may also have a role in rhabdoid tumors. Atypical teratoid/rhabdoid tumor (ATRT) characterized by absence of the chromatin remodeling protein SMARCB1, have an expression profile inversely correlated to the Ezh2 expression. The targeted disruption of EZH2 by RNAi or pharmacologic inhibition strongly impairs ATRT cell growth, suppresses tumor cell self-renewal, induces apoptosis, and sensitizes these cells to radiation with a simultaneous repression of cyclin D1-E2F axis [68]. EZH2 may be a useful biomarker of long-term metastatic risk in women with familial early-stage breast cancer [69]. The p53/p21 inactivation combined with high expression of mitotic cyclins and EZH2 predispose to mitotic entry during S-phase with cells reliant on WEE1, a serine/threonine kinase, to prevent premature cyclin-dependent kinase (CDK) 1 activation. These features are characteristic of aggressive breast, and other, cancers for which WEE1 inhibitor combinations are a promising targeted therapy [70].

EZH2 is now rising as a potential therapeutic target especially in high-risk tumors. EZH2 is highly expressed in medulloblastoma and can be correlated with genomic gain of chromosome 7. 3-deazaneplanocin(a potent EZH2 inhibitor) treatment or RNAi mediated inhibition of EZH2

can suppress medulloblastoma cell growth partially via inducing apoptosis and diminishing sphere forming ability of tumor cells in culture which strongly represses the known oncogenic transformation to neural stem cells [71]. A number of HMTs (Histone methyl Transferases) have been implicated in tumorigenesis and progression of multiple malignancies. Potent inhibitors and ligands for some of these proteins using many high-throughput screening methods have been identified that includes WDR5 and EED, components of MLL and EZH2 methyltransferase complexes [72]. Again HMTases, EZH2 and MMSET, are established to have epigenetic links to oncogenesis by working in coordination. EZH2 function upstream of MMSET by mediating H3K36 dimethylation that is associated with active transcription. With an abundant miRNA network this EZH2-MMSET HMTase axis coordinately plays many functions as a regulator of transcriptional repression, activation, and oncogenesis thus can be signified as an attractive therapeutic target in cancer [73].

Pancreatic ductal adenocarcinoma (PDAC) is characterized by overexpression of enhancer of Zeste homolog-2 (EZH2). The inhibitor of EZH2, 3-deazaneplanocin A (DZNeP; 5 mumol/L, 72-hour exposure) modulate EZH2 expression and H3K27me3 formation and significantly triggered the anti-proliferative activity of gemcitabine. The drug combination reduced the population of cells in G(2)-M phase and significantly amplified apoptosis compared with gemcitabine alone. DZNeP /gemcitabine combination reduced cell migration and associated with increased E-cadherin expression. Furthermore, DZNeP and DZNeP/gemcitabine combination effectively reduced the volume of PDAC spheroids growing in CSC-selective medium and reduced the CD133+ cells population [74]. Though DZNep (3-deazaneplanocin A) show promising anticancer activity, the specific genetic determinants underlying DZNep responsiveness in cancer cells remain largely unknown. TP53 genomic status is critical in influencing DZNep response in gastric cancer. Patients with gastric cancer can be stratified based on their TP53 genomic status through EZH2 DZNep targeting [75]. Tumor-initiating HCC cells are highly dependent on EZH2 for their tumorigenic activity. A short-hairpin RNA and S-adenosylhomocysteine hydrolase inhibitor like 3-deazaneplanocin A (DZNep) induced pharmacological interference with EZH2 might be a promising therapeutic approach to target tumor-initiating HCC cells [76]. PRC2 targets are specifically silenced in some metastatic cancers, and some of them can inhibit angiogenesis. At inhibitory concentration, DZNeP is

harmless for non-transformed cells [77]. In another experiment treatment with DZNep depleted EZH2, SUZ12, and 3MeK27H3 in the cultured humanMantle cell lymphoma (MCL) cells [78]. Inhibition of EZH2 with siRNA and DZNep treatment cancer cell were incurred to apoptosis in addition reduced profoundly in colony-forming efficiency and induction of some squamous differentiation genes in HNSCC. Furthermore in two different xenograft models DZNep attenuated tumor growth, caused intra tumor inhibition of EZH2, and induction of differentiation genes in situ[79].

In Malignant Pleural Mesothelioma Cancer (MPM) overexpression of EZH2 is reported with decreased levels of miR-101 and miR-26a. Global H3K27Me3 levels were decrease by the knockdown of EZH2 or EED, or DZNep treatment, thereby significantly inhibited proliferation, migration, clonogenicity, and tumorigenicity of MPM cells. Common as well as differential gene expression profiles were observed following knockdown of PRC-2 members including EZH2 or DZNep treatment [80]. In leukemic cells DZNep can reactivate target genes CDKN1A and FBXO32 which are silenced by distinct epigenetic mechanisms leading to a loss of the proliferative potential [81].

Babbio et al. proposed that UHRF1 expression was negatively correlated with several tumour suppressor genes and positively with the histone methyltransferase (HMT) EZH2 both in prostate tumours and cell lines. Prostate cancer cells reduced proliferation, clonogenic capability and anchorage-independent growth by knockdown UHRF1. UHRF1, along with Suv39H1 and DNA methyltransferases, contributes to epigenetic gene silencing in prostate tumours. These represents a parallel and convergent pathway to the H3K27 methylation catalyzed by EZH2 to significantly amplify inactivation of tumour suppressor genes [82]. HIC1 (hypermethylated in cancer 1) is a tumor suppressor gene epigenetically silenced or deleted in many human cancers and is involved in regulatory loops modulating p53- and E2F1-dependent cell survival, growth control, and stress responses. HIC1 as the first transcription factor in mammals able to recruit PRC2 to some target promoters through its interaction with Polycomb-like proteins EZH2, EED, and Suz12 and can suitably be targeted in different cancers [83]. Studies identify several potential therapeutic targets for SCLCs, including PARP1 and EZH2 and hypothesize that PARP

and EZH2 inhibition, together with chemotherapy or other agents which demands further investigation [84].

In an animal model the functional synergy in prostate cancers resulting from activation of the androgen receptor, Kras, and Akt, drives three of the most frequently activated oncogenic signaling pathways in prostate cancer. The synchronization of androgen receptor and Kras signaling could elevate EZH2 expression and expand prostate cancer progenitor cells in vivo. So a combine targeting of EZH2 with these signaling factors can be beneficial [85]. Let-7a, a microRNA precursor is frequently downregulated in various types of human cancer including nasopharyngeal carcinoma. Let-7a directly targets Enhancer of zeste homolog 2 (EZH2) in human nasopharyngeal carcinoma cells and thus attenuates nasopharyngeal carcinoma cell growth, inhibits cell proliferation and induces cell apoptosis. These outcomes suggest that let-7a and EZH2 may be potential therapeutic targets for nasopharyngeal carcinoma [86].

OBJECTIVES

1. **To analyses the effect of epigenetic modulator 5-Aza 2`-deoxycytidine (AZA) and Trichostatin A (TSA) on breast cancer cell line MDA MB_231.**

2. **To analyses the transcriptional level of EZH2, K-RAS and ELK-1after the treatment of epigenetic modulator AZA and TSA and to analyses its apoptotic induction.**

3. **To analyses the morphological changes in MBA MB-231 cell after the treatment of AZA and TSA.**

4. **To analyses apoptotic induction by chromatin condensation.**

5. **To analyses the migration property, autophagosome formation and DNA damage of MDAMB-231 treated with AZA and TSA.**

MATERIALS AND METHODS

1. Cell lines and culture

We obtained the MDA MB-231 cell line from the *National Centre for Cell Science (NCCS), Pune, India.* The cells are known to be of epithelial breast adenocarcinoma origin and are triple negative. These were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin sulfate (Invitrogen). A treatment was carried out with *5-Aza 2`-deoxycytidine (AZA)* and *Trichostatin A (TSA),* all purchased from Sigma and added to the regular growth media under sterile conditions for 3 days. The media was changed every 24 hour during treatments. All cells were cultured at a density of 1.0×10^5 viable cells/plate in 6 well culture plates.

2. MTT Assay

To determine the proliferative activity, MDA MB-231 cells were seeded in two 96-well plates at a density (5000-40,000 cells or 10^6 cells) based on the doubling time, with 200 µl growth media (10% FBS) and incubate for 24 hrs in incubator with 5% CO_2 concentration at 37^oC. Cell seeding must be uniform in order to obtain a dose response effect of the drug. The drug of interests *5-Aza 2`-deoxycytidine (AZA)* and *Trichostatin A (TSA)* were diluted at 10 different concentrations in the growth media. In parallel the cells with the solvent control was also treated to assess its effect on cells. After 24 hours existing media was removed and replaced with media with various concentration of drugs and was incubated for 24 or 48 hours at 37^o C. To detect the cell viability MTT working solution was prepared by diluting the stock solution (stock 5mg/ml PBS, PH 7.2) in growth medium without FBS to the final concentration of 0.8mg/ml.100 µl of MTT working solution was added to each well and incubated for 4 hours in CO_2 incubator. After incubation, the media was removed carefully without disturbing *formazan*

precipitate and dissolved in 100 µl of 100% DMSO. An incubation of 15 minutes was carried out in dark and the colorimetric estimation of formazan product was performed at 570nm in a micro plate reader. The data was plotted against drug concentration and non-linear regression curve fitting was performed using *graph pad prism* software to calculate the optimal growth inhibitory concentration (LC_{50}) of the drugs.

3. Isolation of Total Cellular RNA

The total cellular RNA was extracted using TRI reagent (Sigma), following the manufacturer's instructions. On the culture dish 1 ml of the TRI Reagent per 10 cm^2 of glass culture plate surface area was added. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. TRI Reagent is not compatible with plastic culture plates. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 minutes at room temperature. 0.1 ml of 1-bromo-3-chloropropane or 0.2 ml of chloroform were added for per ml of TRI Reagent used. Samples were covered tightly, shaken vigorously for 15 seconds, and were allowed to stand for 2–15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 2–8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).The aqueous phase was transferred to a fresh tube and0.5 ml of isopropanol was added per ml of TRI Reagent used in Sample Preparation, step 1 and mixed. The sample was allowed to stand for 5–10 minutes at room temperature and centrifuged at 12,000 ´ g for 10 minutes at 2–8 °C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and RNA pellets were washed by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in Sample Preparation. The sample was vortexed and then centrifuged at 7,500 ´ g for 5 minutes at 2–8 °C. The RNA pellets were briefly dried for 5–10 minutes by air drying. An appropriate volume of nuclease free water was added and mixed by repeated tapping at 25 °C for 10–15 minutes.

4. Quantification of the total cellular RNA

Final preparation of RNA was analyzed using a nano-drop UV spectrophotometric analyzer. It was likely that a standard preparation of RNA should have a 260/280 ratio of 1.7 and a 260/230 ratio of <1.65 which indicates the preparation to be free from proteins and oligo-peptides contamination. Ethidium bromide (EtBr) staining of RNA in agarose gels visualizes two predominant bands of small (2 kb) and large (5 kb) ribosomal RNA, low molecular mass (0.1–0.3 kb) RNA, and discrete bands of high molecular mass (7–15 kb) RNA.

5. cDNA Synthesis and Evaluation

In a 1.5 ml tube 5 µl of Deionized RNAse free water, 1 µl of T_{18}- oligo (1 µg), 6 µl of dNTPs (10mM), 6 µl of Total RNA (3 µg) were added to make a total volume of 18 µl . The tube with the contents was incubated at 65°C for 3 minutes. The tube was snap cooled on ice and 6 µl of Reverse Transcriptase buffer (5X) ,3 µl of DTT, 1 µl of Reverse Transcriptase, 1 µl of RNase inhibitor and 1 µl of RNase free water the following added to make a total volume of the reaction mix 12 µl .The tube was then snap spinned and incubated at 45 °C for 45 minutes. Again an incubation at 90 °C for 5 minutes followed up. After incubation a snap spin was carried out and 30 µl of deionized water was added. The cDNA prepared can now be used immediately or can be stored at -30 °C. The synthesized cDNA was evaluated by performing PCR for one of the house keeping genes such as β-actin. PCR reaction mix was prepared with 5.0 µl of 10X Taq polymerase buffer, 2.5 µl of 10mM dNTP mix, 1.5 µl of 1.5 mM $MgCl_2$, 2.0 µl of Oligo (mActin- Forward) 10 µM, 2.0 µl of Oligo (mActin- Reverse) 10 µM, 1.0 µl of Taq DNA polymerase and 26.0 µl of deionised water to make a total volume of 40.0 µl. Two aliquots of 20 µl of PCR mix was transferred into 2 PCR tubes. 5 µl of cDNA was added in one tube and 5 µl deionized water was added in another. The tubes were snap spinned to collect the contents in the bottom of the tubes and were subject to PCR with the following cycling conditions of ; 92° C for 3 minutes, 30 cycles of 92 °C 30 sec, 50 °C for 30 sec, 72 °C for 30 sec, 72 °C for 5 minutes and 4 °C for forever. The contents were loaded in 1.5% agarose gel and electrophoresed

along with a molecular weight marker. The gel was visualized under UV light and results were documented in a gel documentation system.

6. RT-PCR Analysis

We used total RNA (2 μg) isolated from the cancerous cell lines for reverse transcription and amplification. The primers used for RT-PCR were designed so that there is an intron between the amplified regions to recognize any DNA contamination. We used four sets of primers to amplify EZH2, K-Ras, Elk-1 and β-actin genes having sequence as detailed in Table no.2 for each RNA sample. The PCR were carried out using standard protocols and the DNA was amplified under the following conditions: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and the final extension of 72 °C for 5 min. We then analyzed the PCR products on a 1% agarose gel.

Table. 2 Primers used for RT-PCR Analysis

Serial No.	Primer Name	Sequence	Scale(uMol)	Purification	Length
1	EZH2 Forward primer	5‘TCAAAACCGCTTTCCTGG3’	0.025	DST	18
2	EZH2 Reverse primer	5’TGTCCCAATGGTCAGCA3’	0.025	DST	17
3	K-Ras Forward primer	5'ACTGGGGAGGGCTTTCTTTG3'	0.025	DST	20
4	K-Ras Reverse primer	5'GGCATCATCAACACCCTGTCT3'	0.025	DST	21
5	Elk-1 Forward primer	5'CACTTCTGGAGCACCCTGAGTC3'	0.025	DST	22
6	Elk-1 Reverse primer	5'AGAGGCCATCCACGCTGATA3'	0.025	DST	20

7. Scanning Electron Microscopy (SEM) Analysis

Cells on attaining 70% confluence on 0.2% gelatin precoated 10-mm coverslips in 35-mm petri dishes were cultured. Dishes without treatment were used as controls and were kept for the same period of induction using suitable growth media. After 24 and 48 hours of drug treatment with *5-Aza 2`-deoxycytidine (AZA)* and *Trichostatin A (TSA)*, the dishes containing the coverslips with cells were used for SEM analysis. In preliminary experiments, differentiated adipocytes were detached and lipid was extracted by normal fixation and dehydration procedures used for SEM

.Treatment medium from the dishes was decanted and the cells were fixed with freshly prepared 2.5% glutaraldehyde in DPBS at 4 °C for 3 h. The dishes with fixed cells were washed twice with DPBS and dehydrated with methanol. It was also found that the fixed cells could be stored in DPBS at 4 °C for a maximum of 1 week and dehydrated when necessary. Stored dishes were brought to room temperature and again washed once with DPBS. Coverslips with dishes were then dried in vacuum-assisted desiccators overnight and then stored at room temperature till SEM analysis was carried out. The surface of the coverslip was sputter-coated in a vacuum with an electrically conductive 5 nm thick layer of Platinum Precession Etching Coating system. SEM images were then recorded with a scanning electron microscope at a lower voltage (20 kV) and low vacuum mode with a tilt of 30°.

8. Cell migration assay (Scratch assay)

60-mm dishes were coated with proper ECM substrates for the cell type to be studied by incubating the dishes overnight at 4 °C or for 2 hours at 37 °C without rotation or shaking. The unbound ECM substrate was removed and dishes were blocked and coated with 3 ml of 2 mg ml-1 bovine serum albumin for 1 hour at 37°C. Then the dishes were once washed with PBS and refilled with 3–5 ml of media before plating the cells. For the particular cell type used, the appropriate amount of serum in the medium during the in vitro scratch assay is required to be determined. It is always recommended to use a lower percentage of serum than that used in the growth media to minimize cell proliferation, but just sufficient to prevent apoptosis and/or cell detachment. Sub-confluent growing cells were re-suspended in a tissue culture dish by washing cells twice with PBS, adding versene containing trypsin, and then mixing cells with medium containing serum. The solution was gently pipetted and the dish was rocked to disperse the cells equally. An aliquot from the cell suspension was taken and the cell count was determined using a hemocytometer. Cells were plated onto the prepared 60-mm dish to create a confluent monolayer and incubated properly for approximately 6 hr. at 37°C, allowing cells to adhere and spread on the substrate completely. The required number of cells for a confluent monolayer depends on both the particular cell type and the size of dishes and need to be adjusted appropriately. The cell monolayer was scraped in a straight line to create a ‘‘scratch’’ with a p200 pipet tip.

The debris was removed and the edge of the scratch was smoothed by washing the cells once with 1 ml of the growth medium and then replaced with 5 ml of medium specific for the in vitro scratch assay. To obtain the same field during the image acquisition markings were created to be used as reference points close to the scratch. The reference points can be made by etching the dish lightly with a razor blade on the outer bottom of the dish or with an ultrafine tip marker. After the reference points were made, the dish was placed under a phase-contrast microscope, and reference mark was left outside the capture image field but within the eye-piece field of view. The first image of the scratch was taken. The dish was placed in a tissue culture incubator at 37°C for 8–18 hours. After the incubation dish was placed under a phase-contrast microscope, the reference point was matched; the photographed region was aligned to acquire a second image.

9. Analysis of chromatin condensation

After treatment with drugs, the cells were stained with Hoechst 33342 stain (1 mg/ml) and incubated for 10 min at 37°C and images were taken under UV filter using Epi-fluorescent Microscope (Nikon TE 2000E). Condensed nucleus was counted against total number of nucleus in the field, and the percentages of apoptotic nuclei were analyzed.

10. Cytochemical staining for visualizing autophagosomes

An optimal number of cells were seeded based on doubling time in a 96 well plate and kept for 24 hours in incubator with 5% CO_2 at 37°C. After the incubation period, the cells were challenged with the suspected autophagy modulating factor for 12 hours. Then after incubation, the existing media was removed and 100µl of fresh media containing 1µg/ml of acridine orange was added. The cells were incubated for 15min at normal culture conditions. The media was discarded and washed with PBS and fresh media was added to the cells. The cells were analyzed under a fluorescent microscope using blue filter (495nm) to view the green fluorescence (510-530nm) from free Acridine Orange and red fluorescence (> 650nm) from acidic vesicles (autophagosomes).

11. Comet assay to measure the DNA damage

To water baths were equilibrated at 40 °C and ~100 °C respectively. Than 1% low-gelling-temperature agarose was prepared by mixing powdered agarose with distilled water in a glass beaker or bottle. The bottle was placed in the 100 °C water bath for several minutes and was transferred into a 40 °C water bath. Agarose-precoated slides were prepared by dipping the slides into molten 1% agarose and wiping one side clean. It is best to work in a low-humidity environment to ensure agarose adhesion. Agarose was allowed to air-dry to a thin film. Slides can be prepared ahead of time and stored with desiccant. A single-cell suspension was prepared using enzyme disaggregation or mechanical dissociation. The cells were kept in ice-cold medium or phosphate-buffered saline to minimize cell aggregation and inhibit DNA repair. Using a hemocytometer or particle counter, cell density was adjusted to about 2 × 104 cells/ml in phosphate-buffered saline lacking divalent cations. Slides were labeled on frosted end using a pencil. 0.4 ml of cells into a 5 ml plastic disposable tube.1.2 ml 1% low-gelling-temperature agarose at was added at 40 °C.1.2 ml of cell suspension onto the agarose-covered surface of a pre-coated slide was mixed by vigorous pipetting. Agarose was allowed to be gel for about 2 min. After agarose has gelled, slides were submerged in a covered dish containing A1 lysis solution. Samples were lysed overnight (18−20 h) at 4 °C in the dark. After overnight lysis, slides were removed carefully and submerge in A2 rinse solution for 20 min at room temperature (18−25 °C). The process was repeated two times to ensure removal of salt and detergent. Care was taken for not allowing DNA to renature even briefly (i.e., by lowering pH below 12.3) until after electrophoresis, as this will result in DNA tangling and reduced migration. After these three rinses, slides were submerged in fresh A2 solution in an electrophoresis chamber. The chamber was filled with a consistent volume of buffer that is about 1–2 mm above the top of the agarose. It was ensured that the chamber is level using a bubble leveling device. Electrophoresis was conducted in solution A2 for 25 min at a voltage of 0.6 V/cm. The current was about 40 mA using 20 V. The distance in centimeters was measured between the negative and positive electrodes in the electrophoresis chamber. Slides were removed from electrophoresis chamber and were rinsed and neutralized in 400 ml of distilled water. Slides were placed in staining solution containing 2.5 μg/ml of propidium iodide in distilled water for 20 min. Finally the slides were rinsed with 400 ml distilled water to remove excess stain. Analysis of cells was done by

examining at least 50 comet images from each slide. Analyzing doublets or comets at slide edges should be avoided.

RESULTS AND DISCUSSION

1. Cell viability assay by *MTT Method*

MDA MB -231 cell viability was determined by MTT Assay. Both the AZA and TSA treatment showed decrease in cell viability but at lower concentration of TSA treatment showed higher decrease in cell viability.

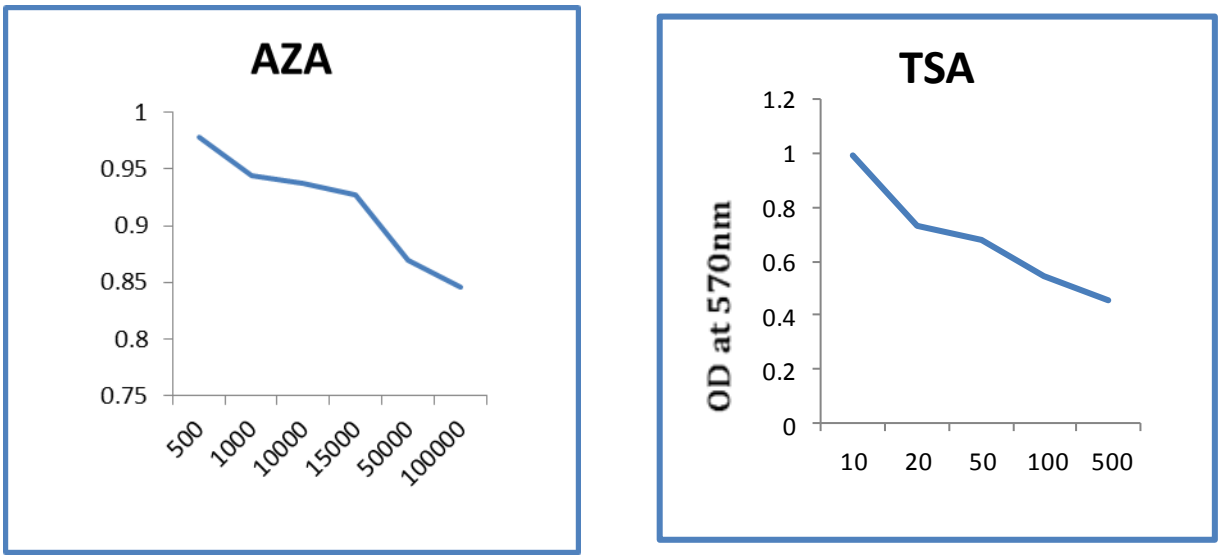


Fig 5. Graphical analysis of cell viability after AZA and TSA treatment

2. Isolation of Total Cellular RNA by *Trizol Method*

The total cellular RNA isolated was isolated following the manufacturer's instructions by Tri-reagent (Sigma).The isolation of the RNA was carried out from the cultured cells of the wells of the six well plates from two wells in a time dependent manner of 24hour and 48 hours. The isolation was almost pure and in good yield as the reading in Nano drop spectrophotometer showed the 260/280 absorption ratio above 1.7 and the 260/230 absorption ratio above 1.65 for all the samples quantified.

3. cDNA Synthesis and *RT-PCR*

Previous studies and the RT-PCR data confirmed that RAS, ELK-1 and EZH 2 were up regulated in breast cancer cell line MDA-MB 231. A time dependent treatment with both AZA and TSA for 24hours showed down regulation of these components.

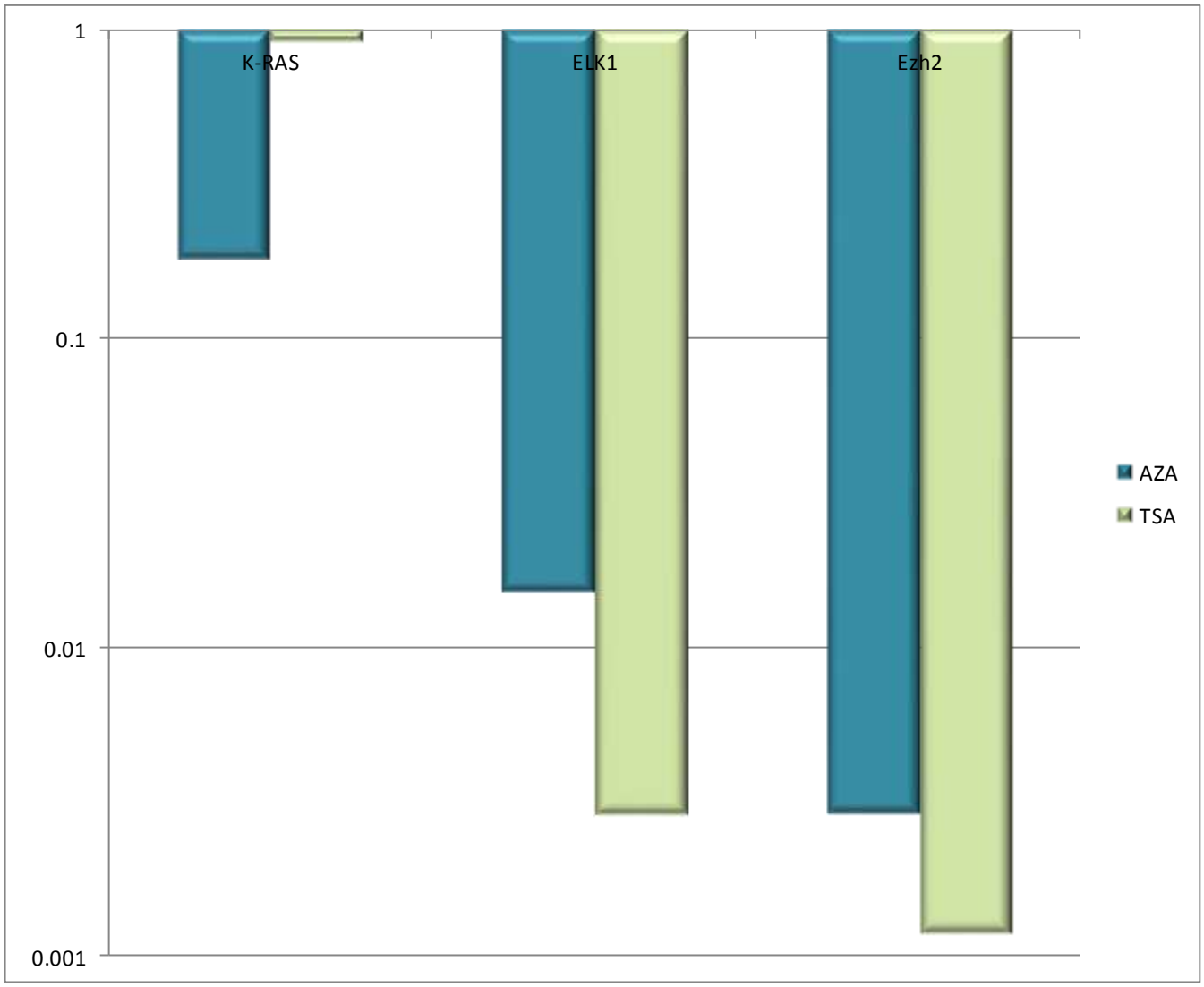


Fig 6. Graphical representation of RT-PCR results for expression level of K-Ras, Elk-1 and EZH2 after AZA and TSA treatment

4. Study of change in cellular morphology by *Scanning Electron Microscopy*

After treatment of the MDA MB-231 cells with AZA and TSA in a time dependent manner for 24 and 48 hours the change in the cellular morphology was observed by a Scanning Electron Microscope. The images suggested that there was a change in the normal cellular morphology of MDA MB 231 cells as they started to attain a round shape suggesting an induction of a mechanism like apoptosis.

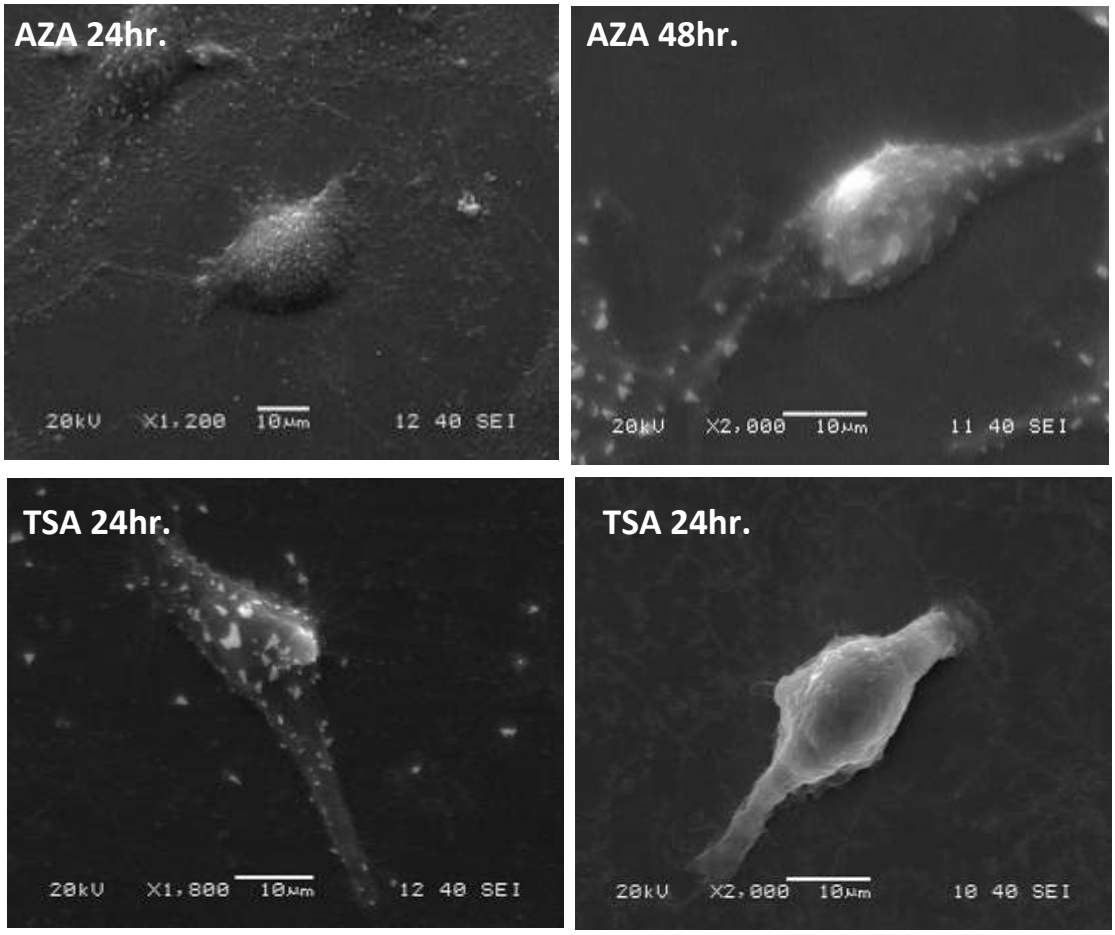
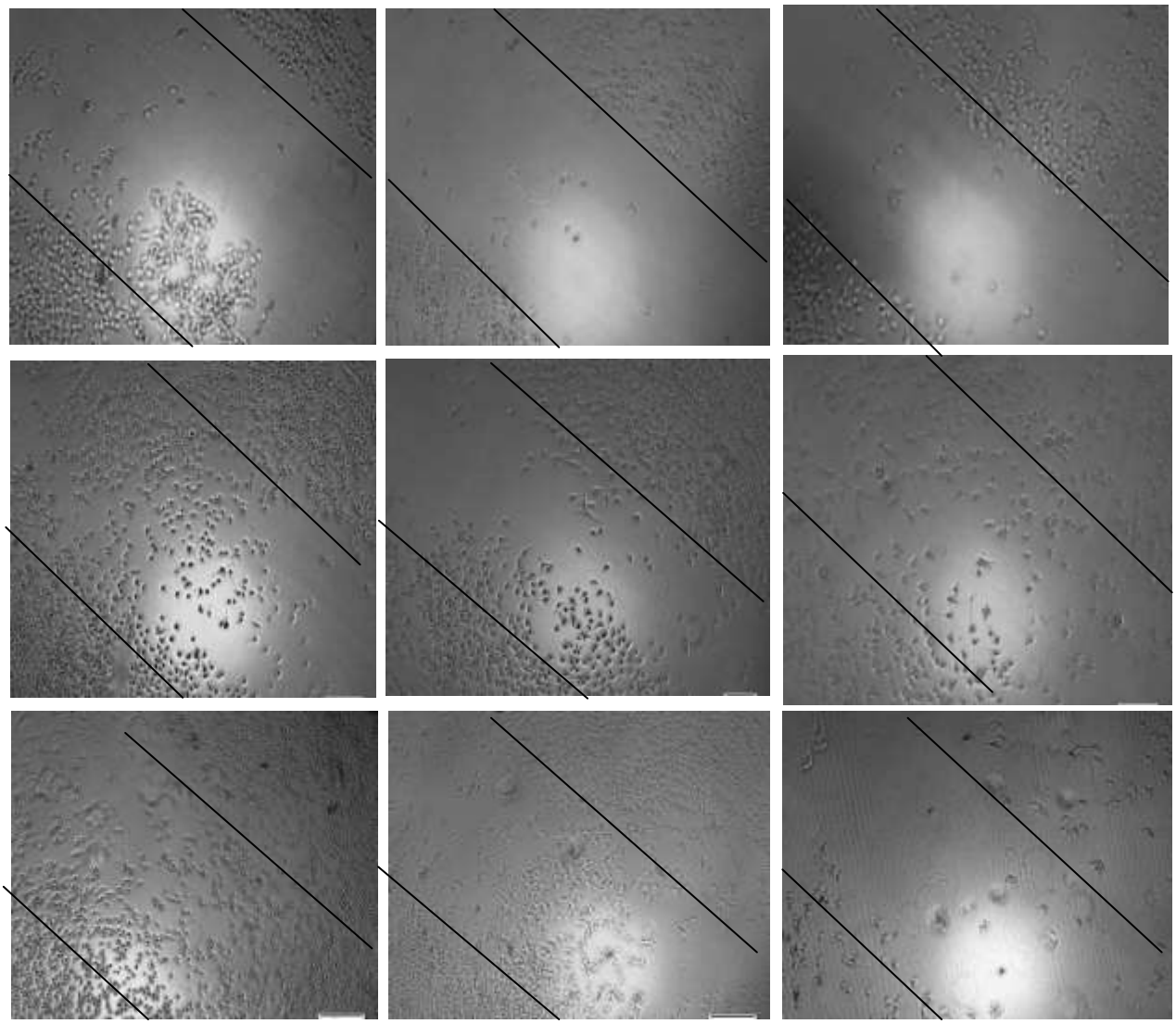


Fig 7. SEM images showing changes in cellular morphology after AZA and TSA treatment

5. Cell migration study by *Scratch assay*

For the study of migratory property of the MDA MB-231 cells after AZA and TSA treatment for 0hr, 24hr, 48hr in a time dependent manner the scratch assay was performed .The results showed that there was more migration of cells towards the scratched area in AZA treated cells as compared to the TSA treated in comparison with the untreated plates taken as control in a time dependent manner.



Control **AZA** **TSA**

Fig 8. Microscopic images showing changes in the migratory property of MDA MB-231 cells after AZA and TSA treatment

6. Analysis of chromatin condensation by *Hoechst 33342 stain*

After treatment with AZA and TSA for 24-48hr in a time dependent manner the MDA-MB-231 cells were stained with ***Hoechst 33342 stain*** and analyzed for the chromatin condensation. The results indicated that there was formation of more condensed structures after treatment with AZA and TSA in a time dependent manner.

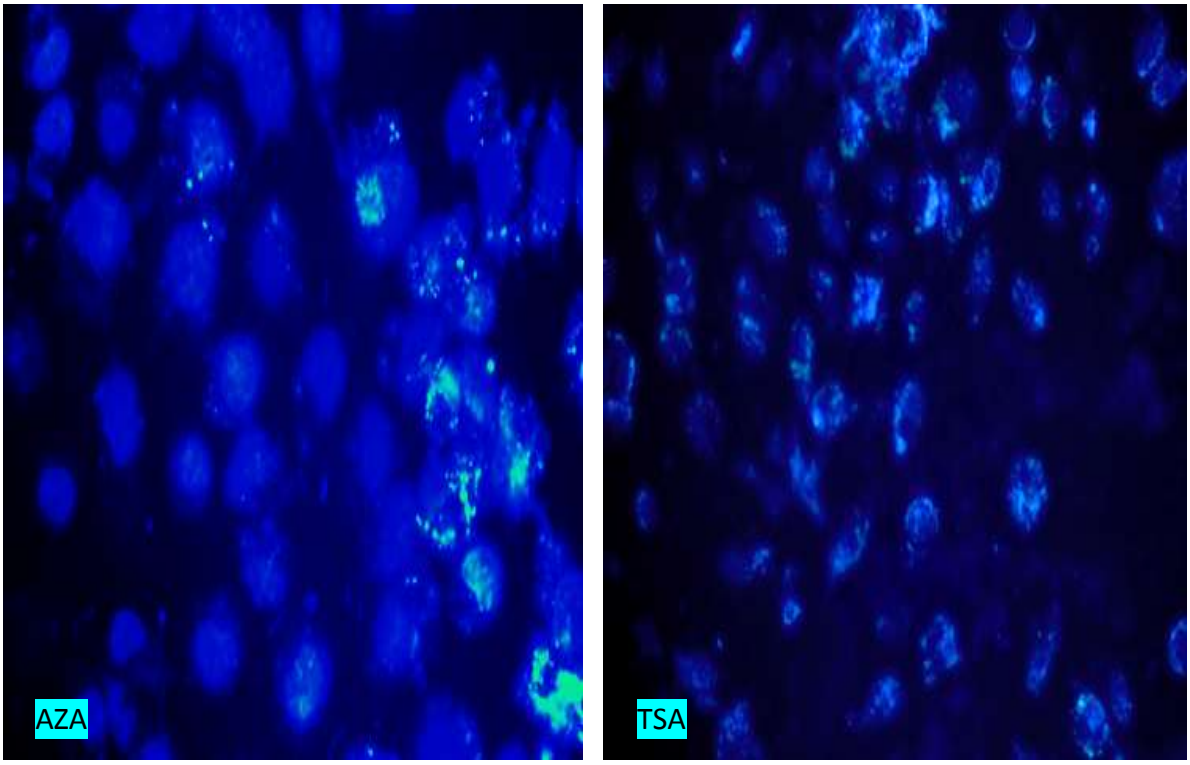
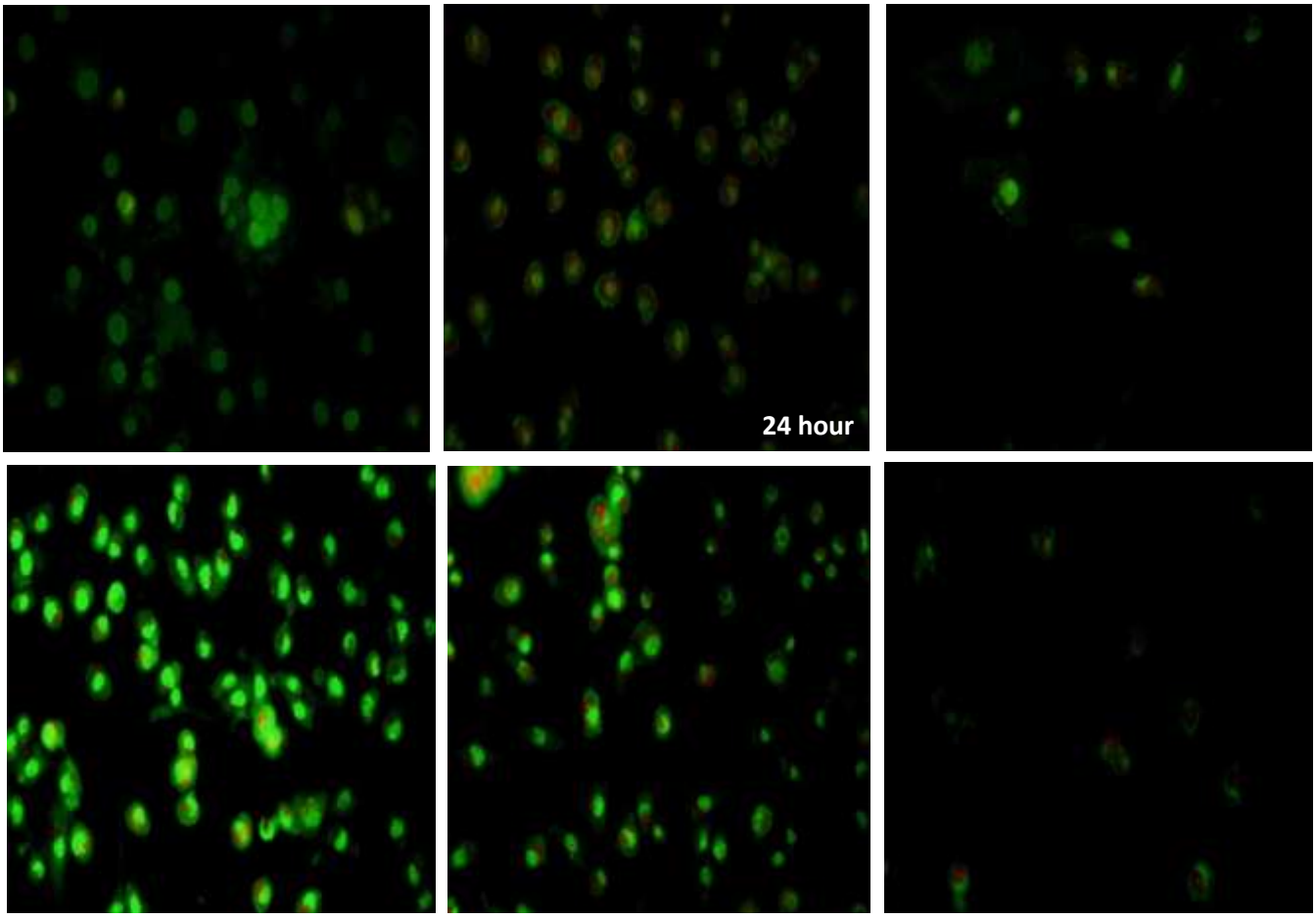


Fig 9. Fluorescence microscopic condensed chromatin images showing after AZA and TSA treatment

7. Cytochemical staining for detecting autophagosomes

The MDA MB-231 cells after the formation of the treatment with AZA and TSA for 24-48 hr. in a time dependent manner were analyzed for the formation of autophagosomes. The AZA treated cells showed greater frequency in the formation of autophagosomes after a treatment of 48 hours. But the TSA treatment was found less efficient in forming the autophagic vesicles.

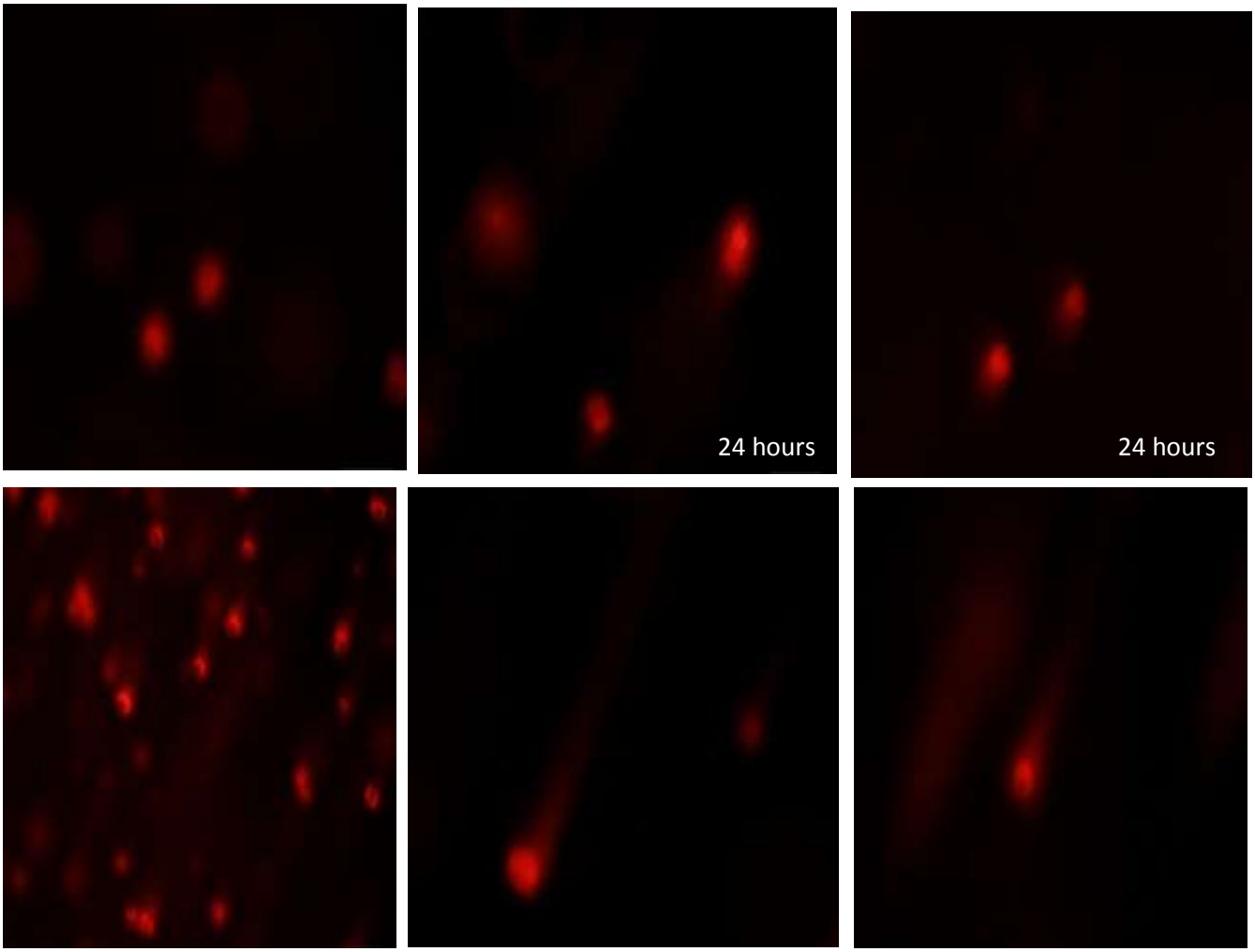


Control **AZA** **TSA**

Fig 10. Fluorescence microscopic images showing formation of autophagosomes after AZA and TSA treatment

8. DNA damage measured by *Comet Assay*

MDA MB-231 cells in culture after treatment with AZA and TSA for 24-48 hr. in a time dependent manner were analyzed for the amount of DNA damage by the comet assay. The results showed significant amount of DNA damage in TSA treatment as compared to AZA and the untreated control cells. The characteristic comet tail length suggested the amount of DNA damaged. In TSA treatment the cells were seen to be having a more dispersed tail as compared to the tails of the AZA treated cells and the control untreated cells.



Control **AZA** **TSA**

Fig 11. Fluorescence microscopic images showing DNA damage after AZA and TSA treatment

CONCLUSION

Apoptosis is a process which is regulated so carefully so that individual cells in a body get it ultimate need. It takes place to maintain a constant cell numbers and to balance the cell proliferation. Additionally apoptosis provide a defense mechanism by which damaged and potentially dangerous cells get eliminated from the body. Similarly it gets activated when cell has undergone DNA damage and as a result of which it induced apoptosis. RAS is one of the most frequently mutated oncogenes in human cancer. Oncogenic mutations in RAS lead to deregulation of several effecter pathways that control cell proliferation, survival, and migration, and thus promote malignant transformation. The best-characterized RAS effecter pathway is the MEK-ERK cascade. It has seen that downregulation of K-Ras and its downstream component Elk-1 has the same effect on EZH2 transcriptional level by treating the cells with AZA and TSA. Therefore it has revealed that apoptotic induction of breast cancer cell line MDA MB_231 is independent of EZH2, K-Ras and Elk-1. The MTT assay showed that TSA has a higher effect than AZA and chromatin condensation showed that TSA treated cells had a greater amount of condensed chromatin than AZA treated cells. The comet assay and condensation of chromatin has revealed that the cells are undergoing apoptotic death but it has no role with EZH2, K-Ras

and Elk-1.

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